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Regulatory and Structural Genes in Migrating Isolated Striated Muscle Cells of Jellyfish

Nathalie Yanze, Hans Gröger, Peter Müller, and Volker Schmid[1]
Institute of Zoology, Rheinsprung 9, CH-4051 Basel, Switzerland

We have investigated, by RT-PCR and *in situ* hybridization, expression of genes encoding regulatory and structural proteins in migrating mononucleated striated muscle cells of the medusa *Podocoryne carnea*. Expression of the three homeobox genes *Otx*, *Cnox1-Pc*, and *Cnox3-Pc*; a specific splice variant of the myosin heavy chain gene (*Myo1*); and a tropomyosin (*Tpm2*) is stable in isolated and cultured striated muscle tissue. When grafted onto cell-free extracellular matrix (ECM), muscle cells of the tissue fragments leave their native ECM and migrate as a coherent tissue onto a host ECM until a stretched cell monolayer is formed. Shortly after the first cells of the grafted isolate have made contact with the host ECM, *Otx* and *Cnox1-Pc* expression is completely turned off in all cells of the graft, including those still adhering to their native ECM. *Myo1* message disappears with a delay while the expression level of *Tpm2* is strongly reduced. However, expression of the homeobox gene *Cnox3-Pc*, a *msh*-like gene, and of the ubiquitously expressed elongation factor 1 α is not affected by the migration process. All genes are reexpressed after 12–24 h, once migration of the cells has ceased. Our results demonstrate that the first few migrating cells induce a change in gene expression which is rapidly communicated throughout the entire tissue. Furthermore, we showed that commitment of striated muscle cells remains stable despite the transient inactivation of cell-type-specific regulatory and structural genes.

Key Words: **gene expression; migration; differentiated cells; *in vitro*.**

INTRODUCTION

Cells and tissues migrate during development, regeneration, and carcinogenesis. To do so, cells have to change their cell–cell and cell–substrate interactions and mobilize a special locomotion machinery which allows them to migrate on extracellular matrix (ECM) or cell layers. Once the target site is reached the appropriate cell–ECM and cell–cell connections are established. These processes require variable cell–cell and cell–ECM interactions, reorganization of the cytoskeleton, and consequently changes in the cell shape. Our knowledge of the molecular control of cell adhesion, spreading, and migration and the corresponding nuclear signaling pathways has considerably improved (Chen *et al.*, 1997; Chicurel *et al.*, 1998; Keely *et al.*, 1997; Klemke *et al.*, 1997; Kheradmand *et al.*, 1998; Yamada and Geiger, 1998). But the consequences of modifications of cell–ECM interactions and cell migration on the expression of genes which are not directly involved in cell locomotion are only rarely described. However, in mammalian cell and organ cultures, it was clearly demonstrated not only that regulatory genes such as Hox genes are influenced by cell–ECM interactions but also that their expression modulates the properties of the ECM (reviewed in Boudreau and Bissell, 1998).

In this study we have investigated the effect of tissue migration on the expression of cell-type-specific regulatory and structural genes in a fully differentiated cell type, the epithelial mononucleated striated muscle cells of the jellyfish *Podocoryne carnea* (Cnidaria, Hydrozoa). The tissue used for jet-pulse locomotion is localized in the bell of the jellyfish, a body part which is known for its excellent regeneration capability. When wounded, the striated muscle tissue migrates over the exposed ECM (mesoglea) and covers it rapidly (Schmid *et al.*, 1976). The striated muscle tissue can be isolated mechanically together with its adhering native ECM (Schmid, 1992) and then grafted

[1] To whom correspondence should be addressed. Fax: 4161-267-3457. E-mail: schmidv@ubaclu.unibas.ch.

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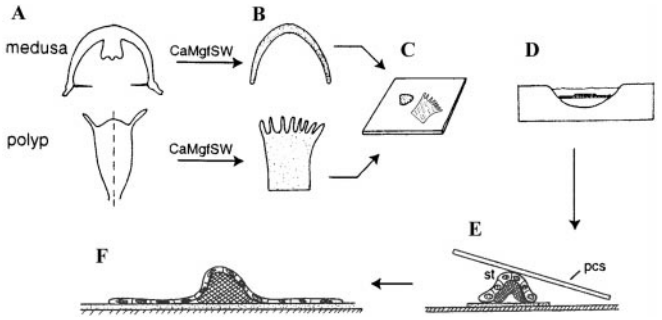

FIG. 1. Schematic drawing summarizing isolation of ECMs and combination experiments of ECMs with isolated striated muscle tissue. Polyps and medusae (A) were incubated in Ca/Mg-free seawater (CaMgfSW). The obtained cell-free ECMs (B) were air-dried on coverslips (C) and afterward transferred to a culture dish (D). Isolated striated muscle tissue (st) was grafted onto the ECMs (E) with the help of a piece of coverslip (pcs). After 12–24 h most cells from the grafted striated muscle tissue have migrated from the graft site and formed a stretched monolayer (F) on the host (stippled area) and on the native ECM of the striated muscle (cross-hatched area) (after Schmid *et al.*, 1992, published by permission of the publisher).

onto cell-free host ECM. Upon grafting, cells leave their ECM and migrate as a coherent tissue from the graft site onto the host ECM. Migration ceases after 12–24 h when cells on the host ECM and those which remain on their own native ECM have formed a well-stretched monolayer (Fig. 1; Schmid *et al.*, 1992).

The structure of the striated myofilaments was monitored by immunohistochemistry using a monoclonal antibody directed against a striated muscle-specific myosin heavy chain (Schuchert *et al.*, 1993). RT-PCR and *in situ* hybridization were used to investigate expression of the striated muscle-specific genes *Cnox1-Pc*, a homeobox gene related to the *labial* Hox gene of *Drosophila* (Aerne *et al.*, 1995); *Otx*, a member of the *Otx* family (P. Müller, personal communication); and *Myo1*, a myosin heavy chain with a striated muscle-specific splice variant (Schuchert *et al.*, 1993), as well as a specific tropomyosin (*Tpm2;* H. Gröger, personal communication). For comparison expression of the homeobox gene *Cnox3-Pc* (*msh*-like; Martinez *et al.*, 1998) and elongation factor *EF1α* (H. Gröger, personal communication) was included. Expression of the structural genes as well as the regulatory genes remains completely stable in cultured and not grafted striated muscle isolates. However, in the grafted isolates, expression of *Otx* and *Cnox1-Pc* is not detectable anymore shortly after the first cells have made contact with the host ECM and started to migrate. With a delay, expression of *Myo1* and of *Tpm2* is also affected while expression of *Cnox3-Pc* and *EF1α* remains unchanged throughout the migration process. All genes are reexpressed once cell migration has ceased.

MATERIALS AND METHODS

Isolation of Striated Muscle and Grafting on Isolated ECMs

Experiments were performed with jellyfish and polyps of *P. carnea*. Culture conditions of the animal are detailed in Schmid (1979). Striated muscle tissue consisting of mononucleated cells and a thin layer of adhering ECM was isolated mechanically by microsurgery from freshly hatched jellyfish (Schmid, 1992). The isolated tissue fragments were cultured in Millipore-filtered (pore size 0.2 μm) artificial seawater at 22°C. Isolation of polyp and medusa (jellyfish) ECM and the grafting process of the striated muscle tissue are described in Schmid *et al.* (1992) and outlined in Fig.1.

Immunohistochemistry

Immunohistochemistry with a monoclonal antibody directed against a protein encoded by a striated muscle-specific variant of myosin heavy chain and staining of the nuclei with 4,6-diamidino-2-phenylindole-2–HCl (Serva, Heidelberg, Germany) are outlined in Schmid *et al.* (1992).

In Situ Hybridization

DIG-labeled RNA probes were prepared according to the recommendations of the manufacturer (Boehringer Mannheim). *In situ* hybridizations were performed according to the protocol described by H. Gröger (personal communication). For the grafted tissues (Figs. 3E–3I) the protocol was modified: fixation was performed in 2% glutaraldehyde in seawater at room temperature for 2 min followed by 15 min proteinase K digestion at room temperature.

mRNA Purification and RT-PCR

mRNA purification was performed on two to five fragments of muscle tissue using the Dynabeads mRNA DIRECT Kit (DYNAL) according to the recommendations of the manufacturer. RT-PCR was done in a one-step experiment combining the MMuLV reverse transcriptase (Boehringer Mannheim) with the *Taq* polymerase (Perkin–Elmer). The cycling parameters we used were 30 min at 42°C, 2 min at 94°C for the reverse transcription step, followed by 30 to 40 PCR cycles of amplification (30 s at 94°C, 30 s at 60°C, 1 min at 72°C). *EF1α* subjected to 25 cycles of amplification was used to normalize the samples. Oligonucleotides used in RT-PCR experiments amplify for *Otx* a 510-bp fragment (*OtxF3*, 5′-ATC AGC GTA TTC ACC AAT CGC AGC-3′; *OtxR3*, 5′-TTG CGG TTC GCA TAC ATT CGA AGG-3′), for *Cnox1-Pc* a 541-bp fragment (*Cnox1-PcF*, 5′-GCA GGT AAC GAG ACT ACT TCG-3′; *Cnox1-PcR*, 5′-GCG TGA TTG GCG GAA ACA GTG-3′), for *Cnox3-Pc* a 496-bp fragment (*Cnox3-PcF*, 5′-CAG CTG CAT ATC AAC TGG AAG-3′; *Cnox3-PcR*, 5′-CAT CCA GTT GAA GCG TTT CAG-3′), for myosin heavy chain a 350-bp fragment which is the striated muscle-specific splice variant *Myo1* (*MHCF1*, 5′-CGA AGC ACG TAA AGG CGC TGA-3′; *MHCR1*, 5′-GTG TTC TTG GCT GGC TTG GT-3′), for the striated muscle-specific tropomyosin (*Tpm2*) a 536-bp fragment (*TPM2F*, 5′-GAG TGG CGA AGA AAA ACT TGG-3′; *TPM2R*, 5′-GCT CTG ATG ATT CTC CTT CCC-3′), and for *EF1α* a 353-bp fragment (*EF1AF*, 5′-ACG TGG TAT GGT TGC CTC TG-3′; *EF1AR*, 5′-TGA TAA CGC CAA CGG CTA CG-3′).

With the exception of the primer pair *TPM2F/TPM2R*, all primer

combinations were designed to span an intron. This allows detection of any putative genomic DNA contamination in the mRNA samples. For all primer sets, a negative control without template was included.

After PCR, amplicons were separated on 2% agarose gels containing ethidium bromide. In some experiments sensitivity of detection was improved by Southern blot analysis. Gels were blotted onto a Porablot NY amp membrane according to the instructions of the manufacturer (Macherey–Nagel). Hybridizations were carried out according to the DIG systems user's guide for filter hybridization (PCR DIG Probe Synthesis Kit; Boehringer Mannheim) with double-stranded DNA DIG-labeled probes amplified with the primer pairs *Cnox1-PcF/Cnox1-PcR* and *OtxF3/OtxR3*. Detection was performed using CSPD (Boehringer Mannheim). RT-PCR experiments were repeated at least twice on three different mRNA preparations. The identities of all bands were confirmed once by direct sequencing.

RESULTS

Gene Expression in Striated Muscle Isolates

Tissue fragments consisting of 100 to 200 mononucleated striated muscle cells were isolated mechanically by microsurgery together with portions of the native ECM. Judged by ultrastructure and immunohistology the isolates cultured in artificial seawater maintain their differentiated state and will not undergo DNA replication (reviewed in Schmid, 1992). To further characterize the muscle isolate, we have investigated by RT-PCR the expression of striated muscle-specific transcription factors. *Cnox1-Pc*, a *labial*-like gene, was shown to be expressed throughout the life cycle of *Podocoryne* only in the developing and fully differentiated striated muscle of the medusa (Aerne *et al.*, 1995); the same is true for *Otx*, a typical member of the *Otx* (*orthodenticle*) gene family (Fig. 3; P. Müller, personal communication), and *Cnox3-Pc* (Martinez *et al.*, 1998), a *msh*-like Hox gene. *Cnox3-Pc* is additionally expressed in early cleavage stages (unpublished). As shown in Fig. 2, all three transcription factors remain expressed in isolated and cultured striated muscle tissue. Since *Myo1* and *Tpm2* could be potential target genes for these transcription factors, we included their expression patterns in our investigation (Fig. 2). To detect transient, short-term, and long-term effects of the isolation process, gene expression was analyzed 15 min, 1 h, 3 h, 6 h, 12 h, 3 days, and 6 days after isolation. The RT-PCR results demonstrate that gene expression is not affected by the wounding (isolation) process (Fig. 2).

To demonstrate that this stability in gene expression is not irreversible, tissue fragments were treated with Pronase immediately after isolation. Previous investigations have clearly demonstrated that Pronase-treated isolates completely transdifferentiate to smooth muscle and nerve cells (reviewed in Schmid, 1992). In this case, the gene expression profile is drastically altered: after 2 days all genes except *Cnox3-Pc* and *EF1α* are turned off (Fig. 2, lane 4).

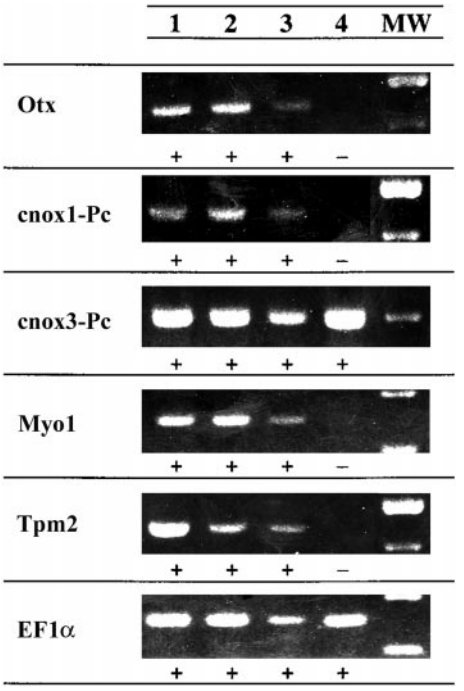

FIG. 2. Gene expression in mechanically isolated fragments of striated muscle tissue not grafted onto host ECM. Lanes 1, 2, and 3 correspond to mRNA samples extracted 15 min, 3 days, and 6 days after isolation and processed for RT-PCR. Samples in lane 4 were activated for transdifferentiation by treating the muscle fragments with Pronase at a concentration of 1.25 mg/ml (from *Streptomyces griseus;* Boehringer Mannheim) for 3 min at 22°C (Schmid *et al.*, 1992). They were processed for RT-PCR 2 days later. 100-bp molecular weight marker is shown in the right lane.

Gene Expression in Striated Muscle Grafted onto Host ECM

To study gene expression in migrating cells, isolated striated muscle fragments were grafted onto air-dried ECMs of polyps (Figs. 1A–1D). Grafting was performed by squeezing the isolate onto the host ECM, thus enforcing immediate contact of many cells with the substrate (Fig. 1E). Upon grafting, striated muscle isolates readily adhere to the ECM and cells start to spread and migrate as a coherent tissue, apparently pulling the neighboring cells from their own native ECM onto the host ECM (Figs. 1E, 1F, 3E, and 3F). Depending on the number of cells which make initial contact with the host ECM, the dynamics of the spreading and the migration process vary among samples. Migration usually ceases 16 to 24 h after grafting, when cells of the isolate form a stretched monolayer (Fig. 1F; Schmid *et al.*, 1992). Because the graft was not kept under culture conditions (Schmid *et al.*, 1999) cells normally did not survive 2–3 days postgrafting and dissociated from the host ECM. Therefore experiments were terminated after 48 h at the latest. Immunohistology showed that the arrangement of

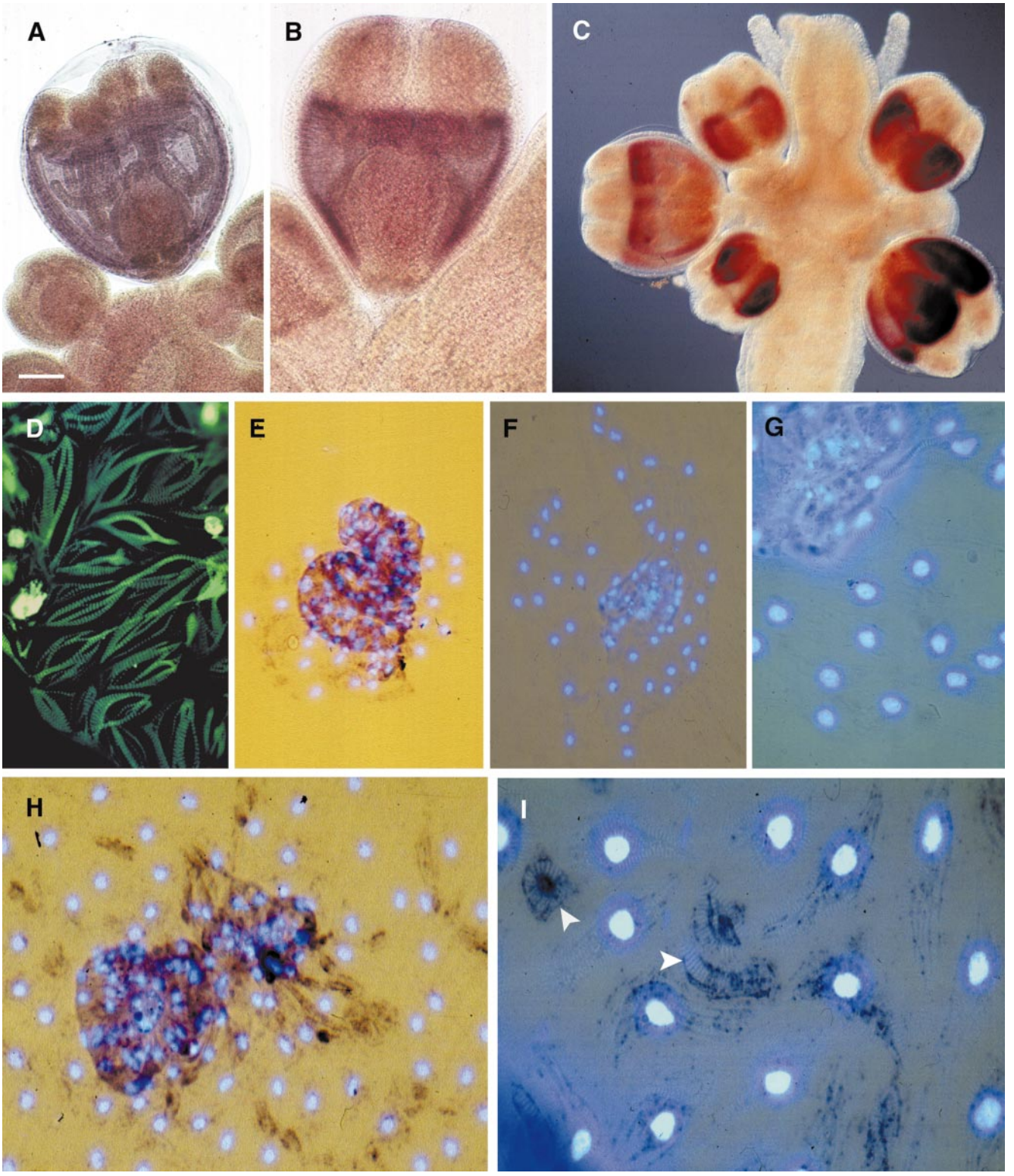

FIG. 3. *In situ* hybridization and immunohistology of developing and grafted striated muscle tissue. *In situ* hybridization during medusa bud development of *Otx* (A), *Myo1* (B), and *Tpm2* (C) demonstrates specific message expression in the differentiating striated muscle tissue of medusa buds of later stages. When grafted on polyp ECM, striated muscle cells of the isolate migrate onto the host ECM. (D) Migrating cells stained with a *Myo1*-specific monoclonal antibody 12 h after grafting. (E–I) *In situ* hybridization of migrating striated muscle tissue with a probe specific for *Myo1* message: (E) 3 h after grafting; (F and G) 9 h after grafting, *Myo1* message has disappeared; (H and I) 24 h after grafting, *Myo1* message is reexpressed in striated muscle cells adhering to both its own native and the host ECM (arrowheads in I indicate *Myo1* message adjacent to striated myofibers). Bar (in mm): 66 in A, 38 in B, 97 in C, 6.6 in D, 8 in E, 13 in F, 6 in G, 7.6 in H, and 3.5 in I.

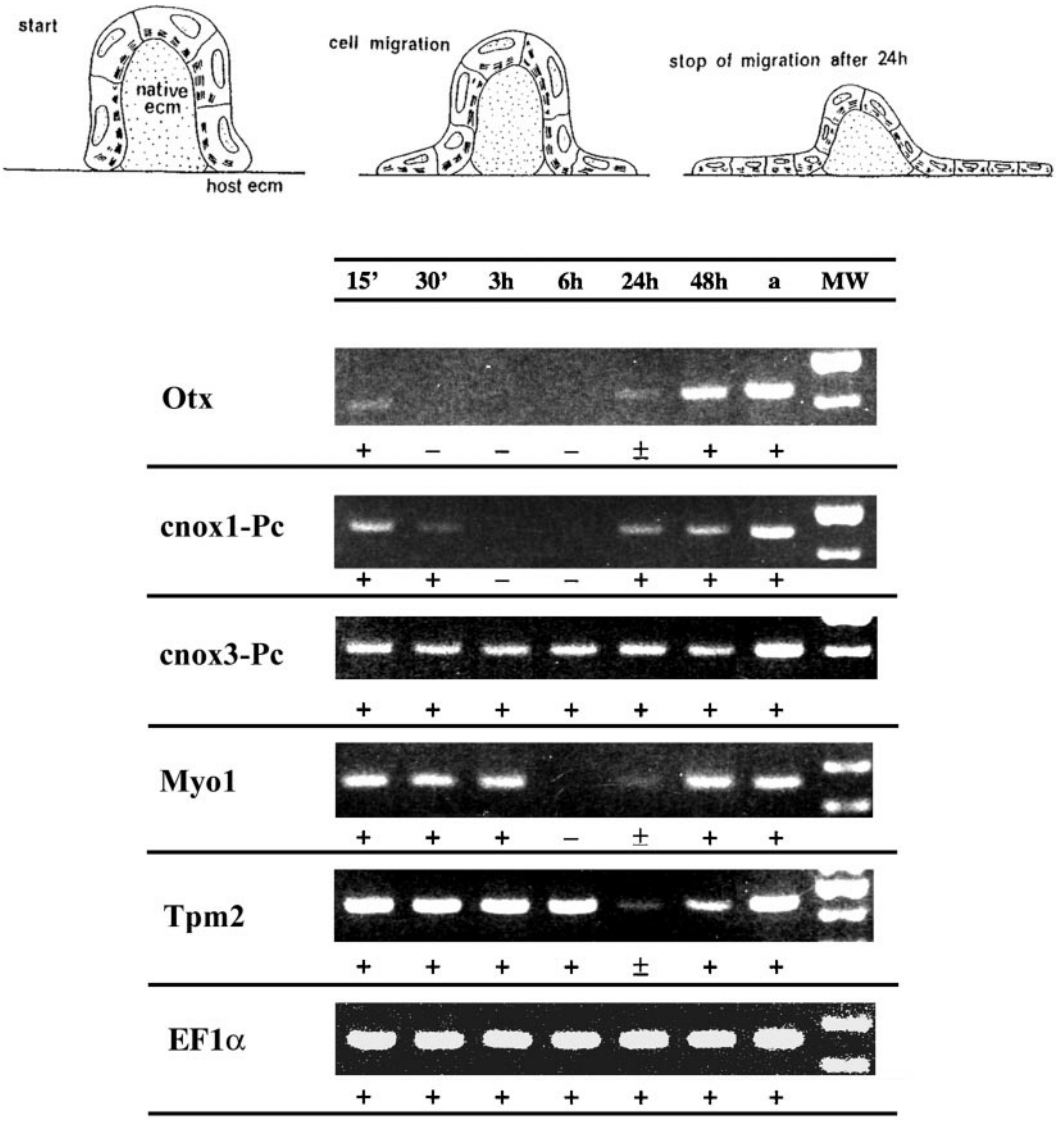

FIG. 4. Gene expression through the migration process as schematized in the drawings at the top. Samples were processed for RT-PCR at the indicated times (top row) after grafting. Lane a, control, strongly squeezed on glass and processed for gene expression after 6 h. MW, 100 bp molecular weight marker. Apostrophe designates time in minutes.

striated myofilaments remained unchanged during the migration process (Fig. 3D).

The RT-PCR results demonstrate that gene expression is rapidly altered in grafted isolates (Fig. 4). First *Otx* and *Cnox1-Pc* messages disappear, followed by the striated muscle-specific splice variant of *Myo1* (Figs. 3F, 3G, and 4). After 3 h the *Otx* and after 6 h the *Cnox1-Pc* messages cannot be detected anymore after 40 cycles of amplification, not even on blotted gels (Fig. 5). Therefore, we conclude that these genes are completely turned off shortly after migration has started and that all the messages are degraded by this time, not only in the cells which have migrate on the host ECM, but in the entire isolate. The level of *Tpm2* message decreases during the migration process but is, in contrast to *Myo1*, always detectable (Fig. 4). *Cnox3-Pc* and *EF1α* expression is not affected by the migration process. Once migration is completed, all genes are reexpressed (Figs. 3H, 3I, and 4). The *in situ* hybridizations, however, indicate that reexpression is not uniform in all the tissues (Figs. 3H and 3I).

To verify if the squeezing, as part of the grafting process itself, and not the cell migration affects gene expression, control isolates were strongly pressed against a glass surface instead of a host ECM. After 10–60 min the tissue detached

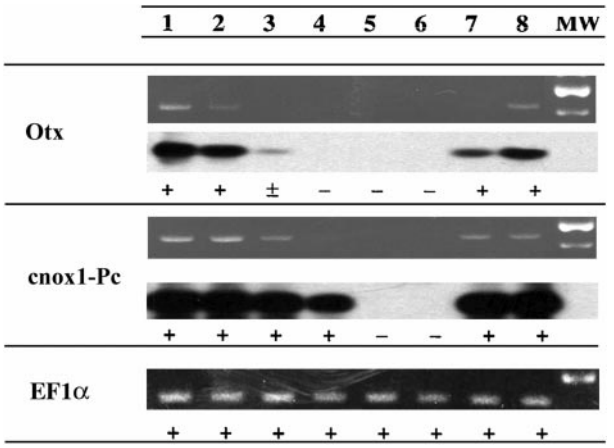

FIG. 5. Southern blot analysis of gene expression through the migration process. Samples were processed for RT-PCR at the indicated times and the corresponding gels were blotted. Last lane shows molecular weight marker. Lanes 1 to 8 correspond to mRNA extracted after 15 min, 30 min, 1 h, 3 h, 6 h, 12 h, and 24 h after grafting.

from the glass and then looked like the ungrafted control. These muscle isolates were processed for RT-PCR: compared to the ungrafted controls (Fig. 2) this strong squeezing procedure showed no effect on gene expression (Fig. 4, lane a). Furthermore, tissue samples which were very gently grafted and thus made only few cell contacts with the host ECM produced the same results as described above. Therefore we conclude that the changes in gene expression are not induced by the grafting method itself; they could be caused by differences in ECM qualities between the native medusa ECM and the host polyp ECM. This alternative was tested by grafting muscle fragments on the native medusa ECM. However, gene expression in isolates grafted onto polyp or medusa ECM looked the same (Fig. 6).

The Need for Continuous Signaling to Repress Gene Expression

The repression of muscle-specific genes, not only in the few migrating cells making contact with the host ECM but in the entire tissue, was unexpected. To test the need for continuous signaling from the migrating cells on the host ECM to the cells adhering to the native ECM the latter were removed from the graft site 3 h after grafting (Fig. 7, lane gr). The removed tissues rounded up and looked indistinguishable from the nongrafted controls, whereas cells remaining on the host ECM quickly stopped further spreading. To study the dynamics of *Otx* and *Cnox1-Pc* expression, the removed tissues and the remaining cells on the host ECM were processed after 30 min, 3 h, and 24 h. RT-PCR results showed that both genes were already reexpressed in the removed free-floating parts 30 min after the tissue was isolated. In the cells remaining on the host ECM, *Otx* message reappeared after 3 h and *Cnox1-Pc* after 24 h (Fig. 7). The results demonstrate that migration is constantly required to keep genes repressed.

DISCUSSION

This investigation demonstrates that during the migration of fully differentiated mononucleated striated muscle cells of the medusa *P. carnea*, genes which are involved in defining the differentiated state are reversibly repressed without inducing changes in cellular commitment.

Why Do Cells Migrate from Their Own ECM to the Host ECM?

In the grafting process, the cells located at the wound edge of the isolate are brought into direct contact with the host ECM. Although these cells adhere to their own native ECM, they seem to prefer the stretched host ECM as an adhesion and migration substrate. The same tissue migration behavior is observed in the *in vivo* regeneration process in which the striated muscle and other epithelial tissues migrate on the exposed ECM and quickly close the wound (Schmid *et al.*, 1976). An increasing number of recent studies have demonstrated that cells generate forces against

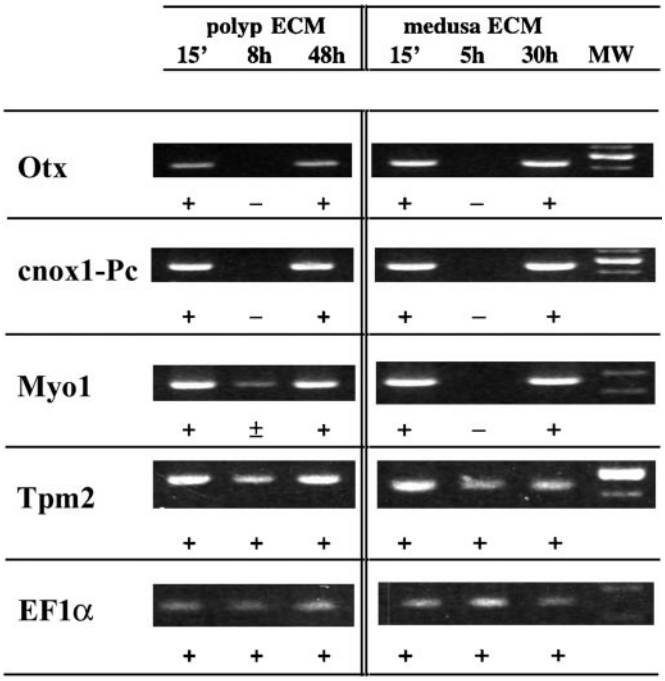

FIG. 6. Gene expression in striated muscle cells grafted onto polyp or medusa ECM. Samples were processed for RT-PCR at the indicated times (top row). MW, 100 bp molecular weight marker. Apostrophe designates time in minutes.

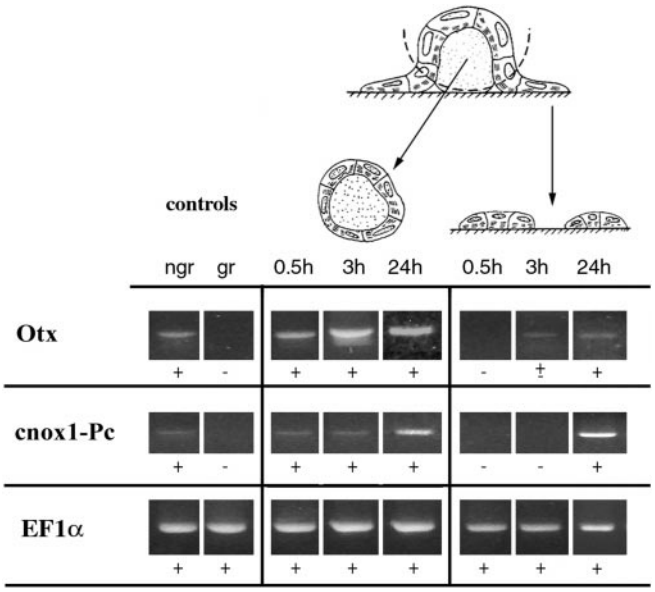

FIG. 7. Gene expression in cells experimentally removed from the grafted isolate and cells remaining stretched on the host ECM. Control shows gene expression in nongrafted muscle tissue (ngr) processed 3 h after isolation from the jellyfish and grafted muscle tissue (gr) processed 3 h after grafting. The scheme depicts the experimental procedure. 3 h after grafting, the cells which were still in contact with their native ECM were separated from the rest of the tissue, which was already in contact with the host ECM, using micropipettes. Both parts were processed after 30 min, 3 h, and 24 h.

the adhesive substrate and that the mechanical stiffness of the ECM affects spreading and motility (Choquet *et al.*, 1997; Pelham and Wang, 1997). Because the host ECM was air-dried its stiffness is higher than that of the malleable native ECM (Fig. 1) and this could influence the migration process. However, it is quite possible that cells will migrate onto any unoccupied ECM as long as its mechanical stiffness can support the migration process. Because the striated muscle cells are tightly connected by desmosomes and gap junctions the migrating cells on the host ECM will pull their neighboring cells onto the host ECM, thus keeping the migration process going on until all available cells have been recruited.

Cell Communication within the Tissue

Within a short time after grafting *Otx* and with some delay *Cnox1-Pc* expression cannot be detected anymore by RT-PCR. Given the sensitivity of the method (40 cycles of PCR followed by Southern blotting) we conclude that less than 1 h after grafting, the *Otx* message has disappeared in all cells of the grafted isolate (Fig. 5). At that time very few cells (<10%) have spread and started to migrate. Therefore, we assume that the first cells which make contact with the host ECM deactivate specifically these genes and signal through their neighboring cells to the rest of the tissue. When the flow of repressive signals from the migrating cells is interrupted as was shown in experiments in which the cells on the native ECM were removed (Fig. 7), gene expression is quickly restored in the tissue. Therefore we concluded that the signals that migration generates are constantly required to keep genes repressed in the entire tissue.

It seems plausible that spreading and migrating cells transfer mechanochemical signals to the neighboring cells which could affect the structure of the membrane, activate stress channels, alter the cytoskeleton, and thus result in a change of cell shape. Every one of these alterations can induce signaling cascades affecting transcription (reviewed in Boudreau and Bissell, 1998; Chicurel *et al.*, 1998; Galbraith and Sheetz, 1998). Preliminary experiments using different cell adhesion and spreading inhibitors of the PKC signaling pathways, like Calphostin C (10 to 100 nM; Vuori and Ruoslahti, 1993; Rodriguez *et al.*, 1997; Chen *et al.*, 1997) or wortmannin (inhibitor of PI(3)K,10 to 100 nM; Keely *et al.*, 1997), and the inhibitor of the Rho family-controlled translocation of NFκB to the nucleus by *N*-acetyl-L-cysteine (10 to 50 mM; Kheradmand *et al.*, 1998) showed no difference in spreading, migration, or gene expression between treated and untreated grafts. The fact that even severe wounding stress, as was exemplified in the strongly squeezed and nongrafted controls, had no effect on the gene expression pattern indicates that the signaling mechanism is highly regulated and specific.

Why Are the Striated Muscle-Specific Genes Repressed in Migrating Cells?

It is evident that cell locomotion requires changes in the expression of genes involved in adhesion and in the architecture of the cytoskeleton. However, it is difficult to understand why genes involved in defining the differentiated state of the muscle are also affected by migration. There is no obvious reason for this unless the differentiated state of the striated muscle is directly controlled by cell–ECM interactions which are modified during migration. We assume that once migration has ceased, the initial cell adhesion complex is reestablished. This would then activate the corresponding signal cascades for the reexpression of striated muscle-specific genes. Furthermore, housekeeping genes like *EF1α* or actin (not shown) or the homeobox gene *Cnox3-Pc* are not affected by cell movement, which is in favor of a cell–ECM interaction-dependent control of the striated muscle-specific genes.

Despite the fact that the striated muscle-specific homeobox genes *Otx* and *Cnox1-Pc* are completely turned off, the differentiated state does not change. This indicates that additional events are needed to induce this step.

Our experiments clearly show that expression of cell-type-specific homeobox genes in a fully differentiated cell is reversibly repressed during migration onto a cell-free ECM.

Additionally, in our system, silencing of cell-type-specific gene expression does not necessarily lead to a change of the cellular commitment as proposed by Okada (1991). It appears that transdifferentiation needs further elements and we regard the DNA replication cycle as a key player which is needed to open new differentiation pathways.

What Is the Biological Meaning of Our Observations?

Cell and tissue migration is an early process in regeneration and there is good reason to believe that across the phyla not all the signaling pathways are conserved. In our model, in which there is no circulatory system for distribution of signaling molecules like cytokines or stress hormones, presumably different signaling pathways have to be used. Based on our observations we assume that cell–cell and cell–ECM interactions in the wound healing process generate the activating signal which spreads fast and reaches cells located even far from the wound site. Although the activating pathway possibly includes elements specific for this lower invertebrate system the cell–substrate interactions seem to be highly conserved in the phylum Cnidaria (reviewed in Schmid and Reber-Müller, 1995).

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